**Editorial comments**

**Thank you for your patience. Please accept again our sincerest apologies for the unusual long delay in delivering the reviews of your manuscript. We have received three additional thoughtful reviews of your pre-print manuscript entitled "Preserving microsatellites? Conservation genetics of the giant Galápagos tortoise." All three reviewers agree that this new version of the manuscript has improved compared to the original version and hold the potential to make a valuable contribution to the field and practices in conservation genetics. I do agree with them.**

Thanks for this positive assessment. We are happy to see that two newly invited reviewers have essentially validated our response to the first round of review, and agree with our main message.

**However, the three reviewers still raise important issues that need to be consider carefully before I can make a recommendation of this manuscript. Reviewer #1 especially has provided a thorough and very critic assessment of the analyses of the transcriptomic data provided in this MS and raise serious issues about the limitation of the sampling of individual tortoises as a demonstration that genomic data contradict previous results obtained with more classical markers. I do agree with the reviewer assessment, and I suggest that the authors tune down their comparison of the transcriptomic data with previous studies.**

Our goal was not to compare our transcriptome data with earlier studies, but rather point to a discrepancy between the two bodies of data, which requires clarification. Details on published mtDNA and microsatellite analyses were absent from the first version, and have been included in the revised version on request from the reviewers.

The same applies here again. Following Reviewer 1's suggestion, we re-examined the mtDNA + microsatellite analysis published by Russello et al. (2010). We show that our sample is not particularly enriched in hybrid-like genotypes, compared to the 156 individuals studied by Russello et al. (2010), thus addressing the reviewer's concern. We also added a new analysis – maximal pairwise F – which is robust to the possible presence of "hybrids" in our sample and corroborates our main analysis. Incidentally, our re-analysis suggests that the level of congruence between markers and methods, and therefore the strength of population substructure in *C. nigra*, have been overestimated by Russello et al. (2010).

**As pointed by the three reviewers a more measured discussion would have merit and could make a significant contribution if it would cover the topics related to taxonomic and conservation units (e.g. population, (sub-)species, management units) since this is at the heart of the argumentation, why genomic data are now required to define those units and assess their evolutionary trajectories in a more insightful and reliable way.**

We agree and have expanded the discussion to cover these topics more broadly. In particular, we put more emphasis on the importance of discriminating between ancient, natural and recent, human-induced gene flow. We also discuss the importance of taxonomy and knowledge of the amount of gene flow between populations in conservation practice. The title and abstract of our manuscript were also amended following suggestions from the reviewers.

**Reviewer #1 implies that some genomic work is currently ongoing on the Galapagos giant tortoise. I suggest the authors to look into that, since the management plan of the Galapagos tortoises states that NGS data are underway.**

We could not find any published article, document or website confirming this statement. The Giant Tortoise Restoration Initiative 2014-2018 plan mentions eight distinct actions of conservation in *C. nigra*, none of which involve genome sequencing. We did find, however, a recently posted preprint manuscript relying on the existing taxonomy and population model, and aiming at "recreate" so-called "extinct lineage" (http://www.biorxiv.org/content/early/2017/05/27/143131). Our call for large-scale population genomic analyses in *C. nigra* is, we believe, more than ever needed.

**I recommend that the authors consider carefully the comments and suggestions of the three reviewers, and address the issues raised in a satisfactory way before I can make a recommendation.**

We hope the new version will be suitable for recommendation by the PCI Evol Biol.

**Reviewer 1's comments**

**I thank the authors for removing Fit as a proxy measure of species divergence and acknowledgement that the negative Fit in the previous version was “troublesome”. The replacement with F does not improve the figure. The attempt to refocus the manuscript on the lack of loci currently used in management plans, rather than insist that microsatellites are not useful markers is a major improvement.**

Thanks for these comments.

**However, I still have reservations about the usefulness of this letter.**

**1) Samples of mixed ancestry:**

**[citing our previous response] The reviewer considers an interesting possibility, which is that there would actually be substantial population structure in C. nigra, but that our sample would not reflect it because it would by chance consist in individuals of "mixed ancestry". We, of course, cannot formally reject this hypothesis.**

**The data on microsatellites and MtDNA does not show “substantial” population structure, but some. At least 2 of the 5 samples are of mixed ancestry see Russell et al. 2010 & Russell et al. 2007 and tables provided below. The samples are not a random selection of populations, but a selection of zoo animals who’s origin was initially unknown. In Russell et al. 2007, only 12.2% of captive individuals were determined not to have MtDNA and micostat data that were consistent- which are 3 of the 4 transcriptome samples. A larger dataset in 2010 reduced the q values of the same samples but now 2/4 were consistent, and a larger analysis of multiple samples Garrick et al. 2014 estimated that q values below 0.8 were considered to be F1 or backcrosses.**

**ZUZ1= Santa Cruz, with a q below 0.768 but now consistently assigned across datasets**

**ZUZ10= PBL with a q of 0.330, indicating hybrid origin but consistently assigned across studies.**

**ZUZ20=PBL or AGO with a q of 0.6 also indicating it is of hybrid origin, has been assigned to two different populations.**

**ZUZ30= assigned to three different populations, VA, VR, and VD with a q of 0.470 indicating it is of hybrid origin.**

**Further to that, ZUZ20 and ZUZ30 potentially from different populations were mixed up in your lab (Loire et al. 2013). The fifth sample is from C. becki, a population elegantly shown to be a lineage undergoing "natural" fusion (Garrick et al. 2014), but this sample is without microsatellite data to compare it to the published datasets. You then go on to discuss specifically the GAE05F and the mixed up sample GA05H, I think this overstates the quality and certainty of your data.**

First, some clarification:

- We did not mix up two samples in the lab; individuals GA05G and GA05H were sampled in the same zoo (A Cupulatta Corsica, France) and the information on which individual corresponds to ZUZ10 vs. ZUZ20 in Russello et al. (2007, 2010), which was not necessary for our purpose, was missing from the beginning.

- We disagree with the suggestion that our sample is not random. The sampled animals were born in the wild, not reproduced in zoos. They are of unknown geographic origin, but their genotypes reflect genuine genetic variation that exists in the Galapagos islands.

- We agree that, since current taxonomical assignment is uncertain (and probably irrelevant anyway, see below) for some of our samples, specifically discussing the relationship between GA05A, GA05E and GA05H is a bit awkward. These sentences were removed.

Now, regarding the Reviewer's main criticism:

Analysing mitochondrial and microsatellite data in Galapagos tortoise, Ciofi, Russello, Poulakakis, Caccone and co-authors have repeatedly argued that the two types of markers are consistent with each other (e.g. Russello et al. 2007, 2010) and indicate "little or no gene flow" among populations (Poulakakis et al. 2015, p2, paragraph 4, last sentence, citing seven papers from this same group). Consequently, these authors have split *C. nigra* in multiple genetically-defined entities that they call species, to which they give distinct scientific names, and which they identify as independent conservation priorities.

Based on this premise, one would expect that a random sample of five individuals carrying divergent mtDNA haplotypes and distinct microsatellite alleles, therefore assigned to distinct species, should exhibit strong genetic structure genome-wide. This is not what we found, which is why we question the existing taxonomy and its use in conservation.

Reviewer 1 comes up with a different explanation: relying on published mtDNA and microsatellite analyses (Russello et al. 2007, 2010), he suggests that several of the five individuals we sampled are by chance of hybrid origin, and therefore not representative of the true population structure in *C. nigra*. Reviewer 1 uses two criteria to call a sample a potential hybrid. One criterion is $q < 0.8$, $q$ being the membership coefficient as defined in Pritchard et al. (2000). The other criterion is inconsistency in species assignment among the different methods used by Russello et al. (2007, 2010) – mtDNA-based assignment, method of Pritchard et al. (2000), method of Rannala & Mountain (1997).

Following Reviewer 1's suggestion we examined Table S1 in Russello et al. (2010). This paper analysed mtDNA and microsatellite data in 156 individuals, mainly from museums. According to

criterion 1 ($q < 0.8$), 73 of the 156 individuals (47%) would be of hybrid origin. According to criterion 2, and conservatively accounting for ambiguous mtDNA-based assignments, 109 of the 156 individuals (70%) would be of hybrid origin. Only 24 (15%) of the 156 individuals analysed by Russello et al. (2010) fulfil the requirement of both $q>0.8$ and consistent population assignment between the three methods.

So our response to this comment is two-fold:
- our sample is not particularly enriched in individuals "of hybrid origin", compared to the set of 156 individuals analysed by Russello et al. (2010); it is representative of the existing genetic diversity and structure in the field;
- the vast majority of giant Galapagos tortoises can not be unambiguously assigned to any of the currently recognized species, according to data from Russello et al. (2010); hybrids are not only common, they are predominant.

This re-analysis of classical markers therefore casts doubts on the existence of species barriers between populations in *C. nigra*, consistent with transcriptome data and the main message of our manuscript. We added a paragraph describing this analysis (line 136-148).

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One issue here is that the high proportion of "hybrids" we estimated from data in Russello et al. (2010) contrasts with the results published in the original paper. For instance, Russello et al. (2010) indicate that 82.6% of the analysed individuals are consistently assigned to the same locality by mtDNA and microsatellite data, implying only 17.4% of hybrids, as recalled by Reviewer 1. How exactly this percentage was calculated is not described in Russello et al. (2010).

We contacted Prof. Russello, who shared his own calls of purebred and hybrid individuals. According to his analysis, only one individual in our sample (ZUZ30) receives conflicting assignments between mtDNA and microsatellites, which is not a significantly higher proportion than the overall 17.4%. This confirms that our sample is not enriched in individuals "of hybrid origin", whichever definition of hybrid one uses.

Prof. Russello clarified that the definition of congruence between markers and methods in Russello et al. (2010) was quite different from Reviewer 1's. In Russello et al. (2010):
- *q* was not considered as a criterion;
- assignments to distinct but related populations from Isabela island were called congruent;
- a match between mtDNA assignment and any of (i) assignment by the Pritchard method, (ii) best assignment by the Rannala-Mountain method, (iii) second best assignment by the Rannala-Mountain method was considered a congruence.
(M. Russello, personal communication)

We concur with Reviewer 1 that this is quite a liberal definition of congruence. For instance, we found that 32 individuals (21% of the whole sample) received a mtDNA assignment that differed from both the Pritchard and Rannala-Moutain ones, but were still considered to be "consistently assigned to the same locality" by Russello et al. (2010) due to a match between mtDNA and the second best Rannala-Moutain assignment.

Our reanalysis therefore suggests that the level of congruence between markers and methods, and consequently the strength of population structure in *C. nigra*, have been overstated by Russello et al. (2010). We did not include this reanalysis in the manuscript.

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**The potential for these samples to be hybrids is not mentioned in Loire et al. 2013. This is important as it limits the usefulness of your already small sample size with regard to discussing species delimitation, it may also be why the other groups, with greater access to samples, have not cited your paper. With the addition of Ballenghien et al. 2017 the authors now report 80% of their own samples have been contaminated by the sequencing centers, I doubt this will add confidence to the tortoise community to utilize these data.**

See above our response regarding the presence of "hybrids" in our sample – not more than in any random *C. nigra* sample, according to data from Russello et al. (2010).

Our transcriptome data have been validated in a number of publications. Both Roux et al. (2016) and figure 1 of the current manuscript demonstrate the potential of our approach to inform on species boundaries in various groups of animals. Ballenghien et al. (2017) showed that contamination, although prevalent, does not significantly affect our published results, and can be accounted for.

Reviewer 1 repeatedly comments on the supposed limitations of our *C. nigra* sample but fails to recognize that similarly size-limited samples provide coherent pictures regarding species delineation in dozens of taxa (our figure 1). Why would it specifically fail in *C. nigra*, and only in *C. nigra*? This is the central argument of our manuscript, of which no rebuttal has been expressed so far.

Speaking about the tortoise community: our dataset includes another species of Chelonian, the European pond turtle *Emys orbicularis*. mtDNA and microsatellite data have revealed strong population substructure between glacial refugia in this species (Lenk et al. 1999 Mol Ecol, Pedall et al. 2010 J Zool Syst Evol Res). Consistently, our transcriptome analysis detected a high excess of homozygous genotypes in *E. orbicularis* (F=0.47), as already pointed out by Loire et al. (2013). This is yet another illustration that our approach has the power to detect population substructure when it exists, tortoises being no exception.

Finally: we do not care whether the Loire et al. (2013) paper is cited or not. What we are concerned about is Galapagos tortoises being arbitrarily sterilized, inbred, translocated from place to place by helicopter, or kept in captivity forever, with much publicity, in the absence of proper scientific justification for such interventions. We modified the manuscript in order to avoid suggesting that our main problem is that our 2013 paper has not been sufficiently cited.

**Many other studies have demonstrated low genetic diversity is in the Galapagos tortoises, your data, with more (>1000) transcriptome loci - show the same low diversity. These data are already published (Loire et al. 2013, Ballenghien et al. 2017), not novel, nor show conclusively more than can be gained Garrick et al. 2014.**

We disagree. Besides our contribution, we are aware of a single study of non-microsatellite nuclear markers in *C. nigra*, namely Caccone et al. (2004). These authors analyzed 4 kb of nuclear sequences and reported very low amounts (~0.1%) of sequence divergence among so-called

"species", consistent with our transcriptome data, as we recall (line 104). Subsequent papers on Galapagos tortoise population genetics, including Garrick et al. (2014), did not comment on such a lack of nuclear sequence differentiation but rather insisted that *C. nigra* populations are genetically well separated as far as mtDNA and microsatellites are concerned (see above and in the ms our reservations about this statement). The fact that genome-average nuclear divergence/diversity in *C. nigra* is extremely low is anything but prominent in the literature, and certainly not considered in *C. nigra* conservation policy.

**It is well established that the tortoises are a recently diverging species group, and from Garrick et al. (2014) view under pd or biological species concepts would be considered full species.**

We disagree. Garrick et al. (2014) identified an episode of massive hybridization between two lineages of *C. nigra becki* called PBR and PBL. Having demonstrated the absence of a species barrier between these two gene pools despite relatively ancient divergence, they logically question the species status of *becki* and its likely "progenitor" lineage *darwini* (p11, end of first paragraph). The Garrick et al. (2014) analysis in no way corroborates the existence of well delineated, multiple species in *C. nigra*. They conclude: "*Our data show that C. becki's PBL and PBR lineages fail to meet the requirements of the biological species concept* ". Garrick et al. (2014) do reiterate previous suggestions by the same group that the various *C. nigra* populations deserve the species status, but add no new argument in this respect.

**The key here, is lack of loci and management measures based on these loci. >From Russell's et al's own work demonstrating a change of q with larger samples indicates there is not enough loci at present, supporting the authors view. I fully concur with the authors, that sterilization, seems extreme, as does the desire to bring back lineages from the dead, a movement to NGS is certainly required for management.**

We are glad to see that Reviewer 1 shares our view on these key issues.

**I do not think this letter is timely nor expresses a new nuance that can be gained specifically from the author's limited transcriptome data.**

Again, the novelty of our contribution is in the multi-species comparative approach, which demonstrates the relevance of the Loire et al. (2013) data to population structure analysis. We show that the *C. nigra* community is probably misled by excessive reliance on a small set of markers, which have been analysed in a biased way (see our discussion on the removal of so-called "hybrids" prior to data analysis, and see above our reanalysis of Russello et al. 2010). Just because not everybody is familiar with our approach, we need to demonstrate its strength by showing that it can generally be trusted despite limited sample size – hence our analysis and figure 1, which are novel.

**2) The utility of summary statistics to compare diversity across species**

**[citing our previous response]** ***Still, please note that the total amount of nuclear genetic diversity we detect in this sample is extremely low, compared to typical multi-species samples.***

**Using a relative summary statistics to compare genetic diversity across species with different historical processes is flawed (Charlesworth 1998). Fst -like statistics are strongly influenced by within population divergence, markers that sample different parts of the genome will therefore provide different levels of differentiation. Relative measures of differentiation are not appropriate if the goal is to compare species with different population history**

**(Charlesworth 1997; 1998). Elevated levels of F can be due to selection or inbreeding rather than reduced gene-flow (Charlesworth 1998; Cruickshank & Hahn 2014). Genetic diversity can be low due to substantial population decline (e.g. bottlenecks, as known to be the case in the tortoises), small founder effects, and or catastrophic events (e.g. volcanic eruptions). The negative Fst values obtained for the Galapagos tortoises even after "contamination correction" (Ballenghien et al. 2017) remains a concern for these data. Using an absolute measure of diversity would be better, as the authors themselves are aware.**

This comment is difficult to understand. X-axis in our figure 1, $\pi_S$, is an absolute measure of diversity. It is very low, lower than measured level of diversity within most species of animals, and much lower than measured level of divergence between any pair of robustly-defined species, as demonstrated by our figure1.

We totally agree that markers sampled in different parts of the genome are likely to yield different values of F, hence our suggestion of increasing the number of loci.

**[citing our previous response]** ***The reviewer's hypothesis would imply that (i) the distinct C. nigra entities have diverged by no more than ~0.1% genome-wide, and (ii) hybridization is so common that hybrids dominate in our random sample.***

**Previous studies confirm at least 2/5 samples are hybrids. Your lab then mixed 2 of the samples, so the actual number of samples that you have that can be traced back to an individual is n=3. The samples are not random but animals that were zoo residents from unknown origin. You have not sampled genome wide but transcriptomes in at least 2 hybrids. In 2013 you state ZUZ01 was from Rotterdam zoo, but this animal was transferred to London Zoo in 2010. It is important there is clear understanding on what the samples represent. If the authors do not concur with the concept of "purebred" and "mixed ancestors" at all in this system, their points would be strengthened by data from samples that the tortoise population geneticist would consider purebred taxonomic units.**

See above our response to a similar comment. Our sample is not enriched in individuals of hybrid origin, compared to the 156 individuals analyzed by Russello et al. (2010). Again, only 15% of these 156 individuals should be considered "purebred" according to the reviewer's criteria. Clearly the problem goes well beyond our sample. There is an obvious need to re-examine taxonomic units in *C. nigra* and what they mean in terms of conservation.

However, following this comment, we performed a new analysis. We considered every pair of individuals from our sample and calculated F for each pair – including the pair consisting of two "purebred" individuals according to Reviewer 1. We found that the maximal F across pairs was 0.12 – a very low value. This indicates that the unexpected position of *C. nigra* in figure 1 cannot be explained by the possible presence of 2 or 3 so-called "hybrids" in our sample – even the two so-called "purebred" individuals show very low genetic differentiation. This analysis appears at line 144-148 of the revised manuscript.

**Yes management plans should be based on more genetic loci, but no, you have not demonstrated that your approach from transcriptome data is an improvement. You have not demonstrated that the management policy is in contradiction with the transcriptome data. You show low genetic diversity (as seen before) and the management policy is designed to maintain the diversity that exists.**

**Management plan:**

**There is certainly improvement by actually citing the management plan and papers on which the authors are criticizing.**

**The paper would be improved with more written about species, population and conservation units. However, the authors state that this is out-with the scope of their letter, yet I disagree, as it is at the heart of the problem. I question whether this is timely (data published 2013) and I am not convinced that there is enough new data or analyses to warrant such a letter at present.**

**So much of this manuscript is about what constitutes a suitable set of markers to delimit species or management units, but the authors have not addressed the taxonomic history of this iconic group or whether that matters (surely all populations are a priority for conservation). I concur with the authors that prudent use of data would be a useful contribution to Galapagos work, and in that, I include prudent use of the author's own limited data.**

**I do not see there is merit in "putting pressure on groups" that have large datasets already, after they have already stated that they are doing NGS data on these samples. I can see the authors feel others should have cited their dataset, but mixing samples in the laboratory, having at least 2 that are have mixed ancestry (which they seem to be unaware of), and the problem they have experienced of contamination at sequencing centers, limits the usefulness of the data. The authors clearly state they are not in a position to do more, I suggest they wait until more data is available or they get more samples.**

1. Regarding forthcoming NGS data in *C. nigra*:

We have no indication that a genome project, or any population genomic project involving NGS, is currently on its way in *C. nigra*. No mention of such a project is made in the website of the Center for Genetic Analyses of Biodiversity, or that of Prof. Caccone's group in Yale University, and no preliminary data is available from public DNA sequence databases, such as the NCBI. The Giant Tortoise Restoration Initiative 2014-2018 plan, released by the Galapagos Conservancy organization, identifies eight distinct goals for the 2014-2018 period, none of which involves genome sequencing or NGS data:
https://www.galapagos.org/wp-content/uploads/2014/05/GTortoiseRI-4-pager-5-2014-small.pdf

Importantly, even if such a project was secretly ongoing, our suggestion is that costly, potentially harmful human interventions should be interrupted until NGS data have been analyzed and *C. nigra* population structure clarified.

2. Regarding RNAseq vs. classical genetic markers:

When we wrote the first version of this manuscript, our point was like: "There are two distinct bodies of data, each with its own strengths and weaknesses, that give very different pictures of *C. nigra* genetic structure; the discrepancy should be resolved, knowing the importance of genetic data in current management." We thought, and still strongly think, that this message is wise, and of importance, hence our submission. We did not mean to go into the details of how the two datasets had been analyzed, and why they do conflict with each other. Our main point was: more data is urgently needed.

Reviewers have repeatedly incited us to provide more details about previously published mtDNA

and microsatellite analyses, to position our contribution in the pre-existing framework, and to comment on the genotypes of the individuals we sampled. We followed these suggestions, and here is what we found:

a- currently defined species in *C. nigra*, including recently defined ones, differ by microsatellite Fst that can be as low as 0.1 (Poulakakis et al. 2015) – the typical level of genetic differentiation between human populations;

b- these ~0.1 figures are obtained after one has removed so-called "hybrids" from the dataset, which is very unconventional and artefactually inflates the estimated differentiation;

c- there is no evidence for genetic incompatibility between *C. nigra* "species", or for local adaptation, and very little connection with morphology;

d- "hybrids" are very common; assignment to "species" can only be done with confidence in ~15% of the individuals;

e- consistency between markers and methods was overstated in Russello et al. (2010).

The first three conclusions were included in the revised version. Reviewer 1 did not comment on these.

So, we now think we at least partly understand the reasons for the discrepancy, which is that mtDNA and microsatellite-based assessment of genetic structure and species boundaries in *C. nigra* has been biased towards over-splitting, compared to standard taxonomic practice, with obvious consequences in terms of management.

Our multi-species comparison independently suggests that current taxonomy in *C. nigra* is questionable and at odds with normal practice, corroborating the above conclusions. So our view now is that both classical genetic markers and RNAseq data agree in suggesting that current management plan might be misled by inappropriate species delineation and underestimation of natural gene flow between populations of *C. nigra*.

3. Regarding the novelty and timeliness of this contribution:

Loire et al. (2013) only briefly discussed the implications of their data regarding population structure and species delineation in *C. nigra*. Their report of very low $\pi_S$ and F was intriguing, but not a decisive argument. Here we show that:

- the low $\pi_S$ and F value are robust to contamination and sequencing/genotyping error problems, and to sub-sampling of individuals;
- both F and total diversity are much lower than expected from a sample covering several species;
- the Loire et al. (2013) sample is not enriched in "hybrids"; it is representative of the existing diversity in the archipelago;
- published mtDNA/microsatellite-based assessment of population structure/species delineation in *C. nigra* has been biased towards over-splitting.

These are novel elements, which we think deserve to be published. Management in *C. nigra* has been, and still is, heavily relying on a population model that can hardly account for the latest data and analyses. It is more than time to let the community and *C. nigra* managers know. People must be aware that the controversy exists, that more data are critically needed, and that important

management decisions are made in spite of the conflicting pieces of evidence.

We modified the manuscript in order to clarify that our main purpose here is to demonstrate the relevance of the Loire et al. (2013) data to species delineation, and consequently management goals, in *C. nigra*.

**Minor points:**

**Lines 66-70 should be modified to make this clearer. The species were described morphologically (Van Denburgh1914) and later confirmed by genetics.**

**Line 76- who died, not "which died".**

**Garrick et al. 2012 is not in the references.**

reference added

**Line 80: Some problems, however, still hamper the task of defining C. nigra conservation units. For instance, mitochondrial DNA and microsatellite data do not fully agree (Poulakakis et al. 2012).**

**I would replace "conservation units" with taxonomic units to strengthen your argument.**

"taxonomic" added

**Line 124 " from 4 to 11 individuals", but says 5 or more individuals in figure legend.**

corrected

**Line 165-174: Should reference Garrick et al. 2014**

**Line 175: several papers exist already on ancient vs modern gene flow. You should rewrite this last paragraph, it is not as well written as some of the comments to the referees.**

**I reiterate from my previous review, a more measured discussion would have merit, highlighting the issues raised, however even if they were to do so, I cannot recommend this letter based on relative summary statistics of limited data, the transcriptome data has been published before, the management plan of the Galapagos tortoises states they are doing NGS, so I don't see what purpose this letter would serve.**

See above our reponses: We could not find any document suggesting that whoever is doing NGS in *C. nigra*. None of the eight objectives identified by Galapagos Conservancy mentions NGS. Criticism of "relative summary statistics" is irrelevant. Our contribution is novel in demonstrating the relevance of the Loire et al. (2013) data to population structure analysis and species delineation.

**Samples used in Loire et al. 2013**
**MtDNA and microsatellites are not consistent on population/species assignment for at least 2/5 samples. ZUZ10 and 20 mixed by authors own laboratory. If GA05H is assigned to PBL or AOG in Russell et al. 2010 this may explain why it was more different to GA05A than other individuals assigned to PBL (Line104-105). Lack of microsat loci, is a problem, but is so is the**

**lack of transcriptomes of individuals with unequivocal population assignment (i.e. with a higher q value than 0.8 for a population assignment the amount Garrick et al. consider to be 'purebreds').**

Reviewed by anonymous reviewer, 2017-07-21 02:31

Review of Loire & Galtier: Preserving Microsatellites? Conservation genetics of the giant Galapagos tortoise

**In this letter Loire & Galtier discuss an interesting and highly relevant topic of using genetic/genomics to understand species' evolutionary biology, particularly in conservation biology contexts. The authors present a re-analysis of recently published transcriptome data, the results of which are discordant with previous studies using microsatellites (upon which the current conservation management strategy is based) and call for additional genome-wide studies to inform conservation management of this species.**

**Having read the revised manuscript and the previous thorough reviews and responses from the authors (as well as revisiting Loire et al. 2013 to fully understand the context of the new results), overall I feel that the authors provided thoughtful responses and concordant revisions to the manuscript. The majority of the initial questions and points of clarification that arose in my mind whilst reading the revised version were discussed in the previous reviews. Thus, I will limit my comments to a handful of suggestions-most of which were brought up in the initial reviews but I feel deserve further clarifications in the manuscript in order for readers to understand the arguments, context, and key take home messages. With these changes, I can support the recommendation of the letter by PCI Evol Biol and hope that this can encourage further discourse of the application of genomics in conservation biology.**

Thanks for these positive comments.

**-Despite the authors' thoughtful responses to comments stating that their key message is that further examination/valuation of giant Galapagos tortoise genomics is needed, as well as their argument is not against microsatellites as markers but rather the limited # of markers used in previous studies, both of these points are absent from the abstract.**

Agreed. We modified the abstract to clarify that the problem is limited number of loci and to call for further population genomic analyses in *C. nigra*.

**-Additionally, I agree with the previous reviewer's comment that the title (which seems to have remained unchanged) is misleading about the issue being with the # of markers vs the type. I wouldn't necessarily withhold recommending the letter if the authors feel strongly about the title, but personally I think the authors could come up with a better fit to accurately encapsulate the points and fuel a healthy discourse.**

We acknowledge that the previous title could be interpreted as an general criticism of microsatellite-based conservation genetics. We certainly do not mean to claim for any level of generality in this piece, which is focused on the Giant Galapagos tortoises. We therefore modified the title accordingly, following the reviewer's suggestion.

**-I thoroughly agree with the previous reviewers' suggestions regarding adding more**

**background information on the conservation context/history, and think the authors did a very nice job of providing that in the revised version. I also think the revisions to the comparative analyses make the findings and relevance clearer.**

Thanks for this comment.

**-I agree that species concepts and taxonomy is a large topic that is beyond this scope of this letter, however I do think it is important to be clear about what species concepts have been used/are suggested in this context. Marine mammal science just published a special issue devoted to this topic of delineating species and sub-species for conservation http://onlinelibrary.wiley.com/doi/10.1111/mms.2017.33.issue-S1/issuetoc The authors may find some content and visuals helpful, in particular : Taylor et al. Guidelines and quantitative standards to improve consistency in cetacean subspecies and species delimitation relying on molecular genetic data**

Thanks for pointing to this interesting series. Taylor et al. 2017 is cited in the revised version.

**-There are several places where I think the authors may want to provide further in text clarification for their methods/arguments to communicate with non-geneticists (i.e., particularly conservation biologists/managers). In particular, the issue of using transcriptome/coding data, which is more constrained, as well as tissue type, is raised and responded to in the reviews, and the authors include citations to other papers where this is discussed to support their methods. I understand the authors don't want to spend a large amount of background when it is better explained/validated elsewhere (and thus this approach is common and acceptable in most situations). However, here the primary goal of this letter is to get the (likely skeptical) reader to consider their argument and not dismiss the results as in err when they are discordant with the previous studies. In particular, to get those involved in conservation programs to pay attention and consider valuing future genome-wide studies, etc. Many conservation biologists, and many conservation geneticists for that matter, are comfortable with markers like microsats, but are not well versed in different NGS approaches (for context, I work as a conservation geneticist using NGS approaches in a government wildlife management agency). Most have learned about using these assumed neutral markers for understanding evol processes, etc., and may immediately dismiss using coding regions like transcriptomes if they don't understand it. Similarly, the low sample # vs. high loci justification...despite multiple studies demonstrating the utility and validity of this (which the authors explain very well in response to the reviews and include citations in text), this remains not well understood and could lead to dismissal of their results from readers who think we cannot learn anything from 5 samples. In sum, I don't suggest that the authors spend a lot of time belaboring the points that this approach is scientifically sound (and I think they do a good job of being transparent with the limitations of the data in the revised version), but I do think a bit more in text explanation would ensure that the audience they seek to reach understands-if the authors really want to have their work impact the conservation of this species, conservation managers need to understand it.**

We followed this suggestion. We added basic information on the statistics we analyze ($\pi_S$, F, line 109-116), and on the power of locus-rich data sets to inform on population history despite small sample sizes, citing the PSMC method and other recent approaches (line 153-158). We also recall that third codon positions of coding sequences are essentially neutrally evolving.

**-Finally, the point discussed regarding the disagreement/uncertainty about whether hybridization/mixing occurred naturally prior to human intervention seems key to the conservation context, and the application of genome-wide analyses to better reconstruct the**

**history (and that we really cannot do this any other way), I think is under-emphasized and would help support the authors' argument for future genome studies.**

Agreed. We added one sentence in the introduction (line 93-96) and one in the penultimate paragraph (line 190-191) to further highlight the importance of this issue.

**Reviewed by anonymous reviewer, 2017-07-13 11:22**

**The authors offer a critique on current Galapagos tortoise conservation practices based upon a reported discrepancy between transcriptome data and mtDNA/microsatellite data. The transcriptome data, generated previously by the authors, suggests the samples used in that study were drawn from a panmictic population (Loire et al. 2013), whereas studies mtDNA (Caccone et al. 2002) and microsatellite data (Ciofi et al. 2002) conclude there is population structure in C. nigra. In the present study, the authors do a comparative analysis of the inbreeding coefficient F versus the log-transformed non-synonymous diversity s. The authors suggest the low value of F estimated for C. nigra indicates low genetic diversity and population structuring compared to other species, questioning the presence of genetically differentiated clusters in C. nigra and, subtly, the need to define subspecies in C. nigra.**

**I think the previous reviewers have already provided a lot of valuable comments that I will not repeat here, so I will focus on sharing my thoughts on the structure and set-up of the paper. In my opinion, the paper has the potential to be an interesting opinion, or even a review, on current conservation practices in Galapagos tortoises and how genomic data should be integrated into it to change it for the better. I therefore really encourage the authors to follow up on this. However, I'm struggling with the present structure of the story. Is the problem the conservation units, the traditional markers, or the "extreme" translocation experiments done? I think there is a choice to be made here by the authors.**

Our point is that "extreme" interventions have been planned because conservation units are improperly defined, and this in turn happens because too few markers have been used. So the three aspects are linked and can only be discussed together, in our opinion.

**To me the issue here does not seem to be the traditional markers themselves or the number of loci. Generally, the problem with traditional markers/low numbers of loci is a lack of power in detecting population structure when gene flow is high or populations are not in migration-drift equilibrium. Marine species seem to suffer from this in particular. Here increasing the number of markers (e.g. SNPs) may enhance more resolution. Of course, the biological relevance of "low but significant" estimates of genetic differentiation comes to mind following this.**

We agree. But in the specific case of *C. nigra*, by adding many more loci we detected less, not more, genetic differentiation. This is an oddity, which we think casts doubts on the prevailing population model in this taxon (see above our response to Reviewer 1).

**Judging from the work of Caccone et al. (2002) and Ciofi et al. (2002) it seems that they do provide convincing evidence for population structure. Even when there is only weak evidence for population structure, it can be better to employ precautionary approach and subdivide a population more than seems necessary to avoid local extinction of potentially undetected population segments. In addition, I agree with the previous reviewers that microsatellites, and mtDNA as well, have valuable traits themselves that make them useful for conservation, e.g. the fast-evolving nature of microsatellites or the use of mtDNA for demonstrating male vs. female-mediated dispersal.**

**From the above section, it also follows that the conservation units don't seem to be the real issue. Delineating conservation units is a challenging thing, and requires careful evaluating of all available scientific data for a species. Ultimately, it is a management decision.**

We agree. A decision of preserving specific populations can be made irrespective of genetic structure. Giant Galapagos tortoises, however, are maximally protected throughout the archipelago. The definition of conservation units in *C. nigra*, therefore, is not relevant to local protection. Rather, genetic data in *C. nigra* are used to make decisions regarding which individuals should be crossed with which, and where they should be relocated. Such interventions would not be justified if *C. nigra* was a single gene pool, or a weakly structured metapopulation. This is why, in our opinion, the definition of conservation units does matter. We added a section discussing these aspects more specifically (line 186-192).

**Judging from the answers of the authors to the previous reviewers, I carefully conclude that the problem seems to be that we can now do genomics at an affordable cost, and it's not being done for the Galapagos tortoise despite its conservation status and current costly management practices heavily based upon genetics. I feel the story can be focussed and streamlined more to fit with this aim. For example, the focus can be narrowed by emphasizing possible genomic consequences of the current management practices (e.g. really focus on inbreeding, potential losses of adaptive potential?) in a "what if we're wrong?" manner using the precautionary principle. I do believe the authors have the potential to write a really important opinion here, but the scope needs to be narrow enough to make the point of the authors clear.**

**As the authors mention in their reply to the first reviewers, they are aware that a full discussion of defining species, sub-species, conservation units and how genetic data fits in here is beyond the scope of the paper. I fully agree with this. However, in the current format that discussion (different subspecies of C. nigra) seems to be at the core of the paper. The authors provide one analysis, the comparative analysis demonstrating a low inbreeding coefficient in Galapagos tortoises compared to other species, and conclude it is unlikely that multiple differentiated gene pools are unlikely and question the need to define sub-species. This makes the story convoluted, because this ties into the classic discussion: what is a species, sub-species, population?**

We tried to make clear why the definition of species and the amount of gene flow between entities (two related concepts) are key to the relevance of current conservation practice in *C. nigra* (line 94-96, 186-192).

**Given the conservation issue of Galapagos tortoises and the fact that genomic resources are available at an affordable cost, I fully agree with the authors and encourage them to follow up on this paper. However, I would recommend to carefully look at the story, evaluate which problem is most important to the authors and restructure the story accordingly. As mentioned before, an opinion paper would be one possibility. Or if the authors are up to it, a review of Galapagos conservation genetics and the need for genomic data.**

We further emphasized the need for genomic data, and added new elements of discussion of conservation priorities in *C. nigra*, hopefully clarifying the message of the manuscript.